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13. ABSTRACT *(Maximum 200*

This ongoing case-control study is being conducted at the Columbia Presbyterian Medical Center (CPMC) and is investigating whether exposures to the environmental and dietary contaminants polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HA) are associated with risk of breast cancer. The study is designed to enroll 100 cases and 100 benign breast disease (BBD) controls from whom blood, biopsy tissue and questionnaire data are being collected, as well as 100 healthy controls from whom blood and questionnaire data are being collected. The study is utilizing biomarkers (PAH-, HA-, and smoking related-DNA adducts) as measures of exposure and a biomarker of pre-clinical effects (p53 muations). During year three, patient recruitment continued and an increased focus was placed on the laboratory aspects of the study.

Progress in patient recruitment has been excellent and is running ahead of schedule with 384 patients currently enrolled. Thus far, a total of 2,400 analyses have been performed on blood samples and 351 analyses have been performed on tissue samples . Descriptive statistical analyses of the data have been carried out. Additionally, samples have been used for several pilot studies and the descriptive analyses for these biomarkers are presented as well.

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FOREWORD

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PI - Signature Date

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A. INTRODUCTION

Breast cancer afflicts one in nine women by the age of 85 and is the second leading cause of cancer death among American women. The incidence has been increasing steadily in the United States to reach 182,000 new cases in 1993. Known risk factors account for only approximately a third of cases. It is likely that environmental factors (including exposures related to lifestyle, occupation and ambient pollution) are contributors, particularly in high risk areas such as the northeastern United States. Environmental contaminants such as the polycyclic aromatic hydrocarbons (PAH), heterocyclic amines (HA), cigarette smoke constituents and organochlorine residues are suspected mammary carcinogens of concern (1-3).

Traditionally, environmental cancer epidemiology has been hampered by difficulties in obtaining accurate data on individual exposures and on individual variation in response to carcinogens. The development of biomarkers has provided a tool that can circumvent these problems by providing individual measurements of the biologic dose of carcinogens, pre-clinical effects and susceptibility to cancer.

The goals of this project are to determine: (1) whether specific environmental exposures are associated with PAH-, HA-, and smoking related-DNA adducts in mononuclear white blood cells and breast tissue; (2) whether these biomarkers are associated with breast cancer case-control status; (3) whether increased carcinogen-DNA adduct levels are associated with the presence of mutations in the *p53* tumor suppressor gene in breast tumors; and (4) to store samples for the piloting of other biomarkers (e.g. *ras p21*, *cyclin D1*, *erbB-2*, DDE, and PCB) related to potential environmental and susceptibility factors in breast cancer.

1. Environmental Exposures of Interest

Polycyclic aromatic hydrocarbons (PAH) and aromatic amines are the two main classes of mutagenic chemical carcinogens that have consistently induced mammary tumors in experimental bioassays, and there is evidence that these compounds may play a role in human breast cancer development (1;3-5). PAH are ubiquitous pollutants found in ambient air as well as the workplace environment, drinking water and food (6). Incomplete combustion of organic material, including fossil fuels, is the major source of PAH, such as benzo(a)pyrene (BP), which is used as a representative indicator of total PAH concentrations (7).

Human exposure to heterocyclic amines comes principally through the diet. Creatine, amino acids, and sugars derived from muscle are important precursors for production of these mutagens (8). Muscle from meat, chicken, and fish produce similar mutagenic heterocyclic compounds, with temperature and time being the more important determinants of their formation during cooking (9).

Most studies of active smoking have found either a small positive association (about 20-30%) or no association with breast cancer (10-16). However, few studies have considered age of onset of smoking. A recent study has, in fact, shown that heavy smoking at an early age (before 16) is associated with a greater risk of breast cancer (odds ratio of 1.7) (10). There have been three reports of an increased risk of breast cancer from passive smoking (17). These results require confirmation (16;17). There is compelling evidence that constituents of cigarette smoke reach the breast and damage DNA through adduct formation (see Preliminary Studies) (1;5;18;19). Recent work has seen a smoking associated risk in women who have a genetic polymorphism in the N-acetyltransferase gene(NAT2 slow) which reduces their ability to detoxify aromatic amines (20). The effect was

greatest in women who began smoking prior to the age of 16.

2. Biomarkers Under Investigation: PAH-, HA-, Smoking related-DNA Adducts and *p53* Mutational Spectra

Biomarkers can be used to supplement questionnaire and monitoring data. Extensive data indicate that most carcinogens, including PAH, HA and cigarette smoke constituents, are metabolically activated to electrophilic species capable of covalently binding to cellular macromolecules. In laboratory animals, the carcinogenic potency of a series of genotoxic carcinogens, including PAH, has been correlated with their ability to form covalent adducts with DNA (21;22). Therefore, carcinogen-DNA binding is widely viewed as a necessary (though not sufficient) event in cancer induction. Adduct measurements can provide sensitive integrating dosimeters for potential mammary carcinogens. DNA adducts can be quantitated by the ^{32}P-postlabeling method which measures a broad spectrum of adducts on DNA (23). PAH-DNA adducts can also be analyzed by immunologic methods either by an Enzyme Linked Immunosorbent Assay (ELISA) or by immunohistochemistry (24-26). Here at Columbia, both methods utilize sensitive antibodies, developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, to quantify recognize PAH-DNA adducts. These techniques can provide a measure of the amount of genotoxic carcinogen that is bound to DNA, often referred to as the biologically effective dose, and can be used as a dosimeter in epidemiologic investigations. In the present study PAH-, HA-, and smoking related-DNA adducts are being analyzed in mononuclear white blood cells from cases, benign breast disease (BBD) controls, and healthy controls. They are also being analyzed in breast tissue from cases and BBD controls.

It has been suggested that mutational spectra in suitable reporter genes, such as *p53*, can reflect exposures to carcinogens that are strongly implicated in carcinogenesis (27-29). The spectrum of mutations found in these reporter genes can be conceptualized as the "fingerprint" left by mutagens that are likely to have contributed to the development of the cancer (28-30). *p53* is a tumor suppressor gene, the inactivation of which appears to play a critical role in carcinogenesis. In sporadic breast cancer, mutated *p53* has been found in approximately 50% of tumors (28;29). *p53* is thus a relevant reporter gene in which to analyze the effects of PAH, HA and cigarette smoke constituents on breast tissue.

Studies of the mutational spectra in breast cancer tumors have shown an increase in G→T transversions in CpG dinucleotides on the non-transcribed strand (28;29;31). G→T transversions appear to occur early in tumor development, and have been detected in all stages of disease, accounting for approximately 20% of all mutations (29;32;33). A similar mutational spectrum has been found in lung tumors for which environmental causes are well known (28;34). Combined with the fact that constituents of cigarette smoke (including PAH) are known to cause G→T transversions, this knowledge has led to the suggestion that environmental factors may be responsible for the mutational spectra found in breast cancer (28;29). In addition, HA are also known to induce G→T transversions (31). A finding of an association between PAH-, HA-, or cigarette smoke constituent-DNA adducts and *p53* mutations in breast tissue would provide biologically meaningful evidence that these contaminants play a role in breast cancer development.

Other promising biomarkers include the oncogenes, *ras p21* and *cyclin D1*, which are often overexpressed in breast tumors, and blood levels of the DDT metabolite (DDE) which has been associated with breast cancer development in some, but not all, studies.

3. Preliminary Studies

In a pilot study by Drs. Perera and Phillips, DNA adducts were detected in breast tissue samples by the ^{32}P-postlabeling method using the P1 nuclease extraction procedure (1). This method detects aromatic adducts including those formed by BP and other PAH. Results were available from 31 specimens, including tumor and/or tumor adjacent tissue from 15 women with breast cancer and 5 healthy women undergoing reduction mammoplasty. Among cases, adduct levels ranged from 1.58 to 10.00 adducts/10^8 nucleotides, with a mean of 4.69 adducts/10^8 nucleotides in tumor tissue, 6.13 adducts/10^8 nucleotides in tumor adjacent tissue and 5.3 adducts/10^8 nucleotides in tumor and tumor-adjacent tissue combined. These values were at the lower end of the range seen in lung tissue of smokers and nonsmokers. Among "controls" adduct levels ranged from 0.43 to 4.41 adducts/10^8 nucleotides with a mean of 2.04 adducts/10^8 nucleotides. Smoking histories were available on the 15 cases. DNA samples from 5 of the 10 smokers (tumor and/or tumor adjacent tissue) displayed the characteristic pattern of smoking-related adducts (a diagonal zone of radioactivity) that has been reported in prior studies of lung cancer patients (35). None of the samples from the 5 nonsmokers showed this characteristic smoking-related pattern. The preliminary data indicated that PAH reach breast tissue and cause genetic damage, and that the measurement of carcinogen-DNA adducts in breast tissue is a useful tool for the epidemiologic study of breast cancer development. These findings have subsequently been confirmed by Li and colleagues (5).

4. Study Design

The current case-control study is designed to include 100 breast cancer cases, 100 BBD controls, and 100 healthy controls. Cases and BBD controls are being recruited from the private practices of Drs. Estabrook and Schnabel at Columbia-Presbyterian Medical Center (CPMC). Healthy controls are currently being recruited from the private GYN practices of Drs. Kelley and Levine at CPMC.

Controls are being frequency-matched to cases on age and ethnic group (African American, Caucasian, Latina). Patients with conditions that are suspected of influencing blood biomarker levels independent of carcinogenesis are being excluded. Exclusion criteria include: prior history of cancer at any site, current pregnancy, breast feeding within the prior three months, and bone fractures within the last six months. Within the BBD study group, patients with diagnoses of benign disease with atypia are being excluded. These diagnoses are associated with an increased future risk for breast cancer and these patients may share common risk factors with the cases.

Blood samples, questionnaire data and pathology reports are being collected from all of the patients; and breast tissue samples are being collected from cases and BBD controls. Blood samples are being fractionated, processed and preserved for the assays to be conducted under this grant and to create a bank of specimens to support future research projects. Under this grant, mononuclear white blood cell (MWBC) samples are being analyzed for PAH-, HA-, and smoking related-DNA adducts; and breast tissue samples are being analyzed for PAH-DNA adducts. Additionally, breast tissue samples are being analyzed for mutations in the *p53* tumor suppressor gene using immunohistochemistry and mutation detection with Affymetric's gene chip technology.

Statistical analyses will be used to test our major hypotheses. Logistic regression analysis will be used to determine if carcinogen-DNA adduct levels measured in tissue and/or MWBC are

associated with case-control status after controlling for confounding variables. Additionally, logistic regression will be used to test the hypothesis that, among cases, mutations within the *p53* tumor suppressor gene are associated with increased carcinogen-DNA adduct levels in tissue and/or MWBC. Finally, using questionnaire data on environmental, occupational and dietary exposures, associations between life-style factors and carcinogen-DNA adduct formation in MWBC and breast tissue will be investigated.

B. BODY OF THE REPORT: PROGRESS DURING YEAR THREE

1. Patient Recruitment, Sample and Data Collection

a. Patient Enrollment

Year three of the research project has been completed. Active patient surveillance programs have been conducted in the offices of collaborating breast surgeons, Drs. Estabrook and Schnabel. Cases and BBD controls have been enrolled by two interviewers under the direction of these two surgeons and their staff. All patients undergoing breast surgery with these doctors were evaluated as potential subjects. Eligible patients were identified and enrolled after the physician recommended surgery, but before surgery was performed. The study objectives and the patient's role in the study were explained to each of the prospective subjects, and interested patients signed a consent form that met DOD and CPMC institutional requirements. Following enrollment, the patient was interviewed and a blood sample was drawn. Blood samples were drawn prior to surgery to prevent confounding of biomarker data by exposures to anesthesia, chemotherapy, hormone therapy, biologic changes associated with the healing process, or post-surgical changes in diet.

Healthy control subjects are being enrolled under the direction of Drs. Kelley and Levine and their staff. Dr Kelley and Levine's GYN practices are in the same building as the CPMC Breast Service and these doctors refer their patients to Drs. Estabrook and Schnabel for breast health care. Further, data on birth date and residential zip code were analyzed from a random sample of each physician's patients and were found to be similar across the physician's practices. Women are approached during routine GYN check ups with Drs. Levine and Kelley and are enrolled into the study. These women sign a consent form, donate blood samples and take part in the structured interview. Healthy control patients are being frequency matched on age and ethnicity to cases.

As a result of these surveillance programs, patient enrollment has occurred at a faster pace than originally anticipated (see Table 1). This enhances the strength and validity of study in two ways. First of all, since we have a wide catchment system and are able to evaluate nearly every patient who enters it, we are better able to assure that we have a representative sample of cases. This over-sampling will also assure that we will have complete data and samples (questionnaire and medical record data and tissue and blood samples) for a total of 300 subjects (100 cases, 100 BBD controls and 100 healthy controls). The additional samples collected through these recruitment efforts will be stored for research projects to be conducted under separate funding. Active subject recruitment will continue until January of 1997.

Table I

Patient Enrollment

CATEGORY	PRIOR TO YEAR 3	YEAR 3	TOTAL Thus Far	Expected Year 4	TOTAL
Total Enrolled Patients	251	133	384	64	448
Cases	83	28	111	25	144
BBD Controls	71	24	95	15	118
Healthy Controls	72	56	128	15	143
Other*	25	9	34	9	43
Unknown**	0	16	16	0	

* includes: benign breast disease with atypia, lobular carcinoma in situ, and rare cancers (e.g. Cystosarcoma phylloides).
** Pathology review is not yet complete.

b. Questionnaire and Pathology Data

Each of the patients takes part in a structured interview that covers demographic variables, reproductive and health histories, diet, residential history, smoking, alcohol consumption, occupational history and environmental exposures. Interviews are conducted prior to surgery during the pre-operative testing procedures. Data from the questionnaires are being abstracted into a computer spreadsheet as soon as the interview is completed. The spread sheet is in a Lotus 1-2-3 WK1 format for simple importation into the SPSS statistics package. Pathology reports and data on receptor, proliferative and clinical markers analyzed by the pathology department (estrogen/progesterone receptor status, *erbB-2*, DNA index, G0-G1, S, and G2-M cell cycle status) are also being collected and abstracted into the same spreadsheet as the questionnaire data. Additionally, information on stage and tumor size is being collected from the CPMC Tumor Registry.

c. Biological Specimen Collection and Storage

Blood samples continue to be collected from subjects and separated into total white blood cell, red blood cell, mononuclear white blood cell, and plasma components. In addition to preserving the blood samples for the assays funded under this proposal, our design called for storing of aliquots for future research. Sample aliquots have been processed and stored in anticipation of future analyses of; 1) organochlorines, 2) plasma vitamin C and E, retinoids and carotinoids, 3) hemoglobin adducts, 4) plasma *erbB-2* extra-cellular domain, 5) plasma *ras* levels, 6) plasma p53 levels, 7) plasma EGFR levels, 8) biomarkers of oxidative damage, and 9) metabolic genotype (NAT2, GSTM1, CYP1A1). These procedures have created a sample bank that will allow future research to be conducted in an efficient and economical manner. Under separate funding preliminary analyses of several of these markers have been conducted to support spin-off studies (see **Complementary Studies** and **Pilot**

Studies).

Due to the small size of many of the lesions, frozen tissue is not available from all of our patients. Samples of DNA (50 ug) from the larger frozen samples are being collected. Paraffin embedded biopsy specimens from cases and BBD controls are being retrieved from the CPMC Pathology Department. Each of these samples is reviewed again by the same pathologist to provide a uniform interpretation of the tissue specimen. Samples from these specimen blocks are being shaved off with a microtome and stored on glass slides (10-20 slides for immunohistochemical analysis) or in plastic vials (for future DNA extraction). One slide from each patient is being hematoxylin and eosin (H&E) stained to provide a histologic reference. In year three, The Pathology Department was slow in releasing tumor blocks to us. However, in the second half of the year this issue was addressed and 120 blocks were reviewed and released to us. We do not expect any further delays in retrieving the remaining blocks.

As a complement to this study we have formed a collaboration with Dr. Marc Citron, formerly Chief of Oncology at Long Island Jewish Medical Center (LIJMC), now Chief of Oncology at ProHealth Care Associates. Dr. Citron has supplied us with fresh frozen and paraffin embedded tissue specimens from breast cancer patients diagnosed between 1991 and 1996. With other available funding these women were contacted, informed consent was obtained and the women were interviewed over the phone. The questionnaire was the same as the one being used with the CPMC women, but had been modified to take into account that the women were diagnosed in the past. The tissue samples are being analyzed for the same biomarkers as the tissue samples collected from the CPMC women. This work will allow us to investigate whether environmental exposures and biomarker levels differ between the two populations.

This archive of paired blood and tissue samples and associated questionnaire data and pathology reports is an invaluable resource that supports our current research and will form the basis of future projects that can be conducted in a timely and efficient manner.

2. Laboratory Component

The analyses of biological samples for carcinogen-DNA adducts and *p53* mutations are ongoing; descriptive analyses for these and other markers are presented below. Interim analyses of biomarker data with respect to case-control status have not been performed since multiple analyses were not factored into the study design and thus interim analyses will increase the Type I error rate. Further, multiple comparisons may bias the analytical process if Type I errors are made during the interim analyses and undue attention to these variables influences later analytical decisions. To minimize the possibility of a Type I error we have not decoded the samples and performed case-control analyses for the funded biomarkers. As described in our study design, case-control analyses will be performed at the end of the study when the datasets have been completed.

We submitted an expanded year two progress report at the end March 1997. That report included all work completed up until that time. Due to the overlap between the reports for years two and three, the current report summarizes the work completed in year two (Oct. 1st 1995- Sept. 30th 1996) and in year three (Oct. 1st – Sept. 30th 1997) (see Tables 2 and 3).

a. Postlabelling Analysis of MWBC

As shown in Table 1, mononuclear white blood cell DNA has been analyzed for the presence

of PAH-, smoking related-, and heterocyclic amine-DNA adducts by ^{32}P postlabelling methods in Dr. Phillips' lab. In years two and three, 75 samples were analyzed (total 150), with an additional 75 samples in progress at this time. In two samples there appeared to be assay artifacts and the results were thought to be unreliable. Descriptive data are presented here on the first 148 samples analyzed for PAH-DNA adducts using the P1 nuclease digestion method. The mean adduct level is 6.16 adducts per 10^8 nucleotides with a standard deviation of 3.20 adducts per 10^8 nucleotides. The data show a log normal distribution, consistent with our prior studies of PAH-DNA adducts in white blood cells. Each of the postlabelling chromatographs was analyzed for a diagonal radioactive zone (DRZ) which indicates the presence of smoking- related DNA adducts. Chromatographs from 25/148 (17%) samples had smoking-related DNA adducts, which is would be expected given the low prevalence of current smokers in the study population. In year three, 53 samples were also analyzed for HA using the ATP-deficient postlabelling method. Heterocyclic amines were not detected in any of the 128 samples tested thus far. If none of the samples in the third batch of MNWBC are positive for HA-DNA adducts we will use the remaining funds allocated for ^{32}P postlabeling to analyze PAH-DNA adducts in the fresh frozen tissues we have received from LIJMC.

The fourth batch of 75 samples will be sent at the end of January 1997 and will be analyzed for PAH-DNA adducts, smoking related-DNA adducts and HA-DNA adducts (see Table 2.).

b. Postlabelling Analysis of Fresh Frozen Breast Tissue

As discussed above, due to the small size of the lesions, it has proven difficult to obtain fresh frozen breast tissue specimens from the enrolled subjects. However, through our collaboration with Dr. Citron at LIJMC we obtained 18 fresh frozen breast tissue samples from 12 breast cancer patients.

Seventeen of the 18 tissue samples were analyzed for PAH-, smoking related-, and HA-DNA adducts using postlabeling techniques in Dr. Phillips' lab (one sample yielded no DNA). Mean PAH-DNA adduct levels were 3.38 per 10^8 nucleotides with a standard deviation of 1.62 per 10^8 nucleotides. These levels are in the range seen in our pilot study and also in a more recent case-control study (5). None of the fresh frozen samples showed smoking related-DNA adducts, which is consistent with fact that none of the subjects was a smoker at the time the sample was taken. HA-DNA adducts were not detected in any of the samples analyzed.

Dr. Marc Citron who maintained the tissue repository has recently left LIJMC and turned the repository over to us for carcinogen-DNA adduct analysis. We are currently inventorying approximately 200 tissue samples and pathology reports, and anticipate sending these samples to Dr. Phillips' lab for adduct analyses.

c. PAH-DNA Adduct Analysis by Immunohistochemistry in Breast Tissue

We have used an immunohistochemistry assay to analyze the paraffin-embedded tissue samples for PAH-DNA adducts. The assay utilizes a sensitive polyclonal antiserum[1], developed in Dr. Regina Santella's laboratory at the Columbia School of Public Health, that is highly sensitive and specific for PAH-DNA adducts. Stained slides are analyzed on a Becton Dickson Cell Analysis System (CAS 200) which measures the Optical Density (OD) of the staining on the slides. The OD results provide a quantitative measure of the amount of antibody staining and thus of PAH-DNA adduct levels. As

[1] Note: in the year two report the antibody was inadvertently referred to as a monoclonal antibody.

shown in table 2. in year two, 15 study samples and 8 breast cancer laboratory control samples were analyzed. In year three, another 27 study samples and 3 breast cancer laboratory control samples were run. In the total set of 42 samples from 33 patients the mean OD of the samples was 0.44 with a standard deviation of 0.11. Another 70 samples are being prepared for analysis.

In response to the reviewer's comments on our previous report, we provide below validation statistics (e.g., sensitivity, specificity and reproducibility) for this assay.

Low Dose Sensitivity: Dr. Santella's work with the 10T½ cells shows that the sensitivity is less than or equal to 1 adduct per 10^6 nucleotides. It should be noted that this determination was made on cells with a single type of PAH-DNA adduct, while human samples contain complex mixtures of PAH bound to DNA. The antibody used in this assay actually has a higher affinity for certain other PAH-DNA adducts (e.g. chrysene diol-epoxide-adducts) than for BPDE-DNA (36). Thus, the true sensitivity in human samples is probably greater than seen in cell culture. Dr. Santella's work has shown that this assay is sensitive enough to detect smoking- related differences in adduct levels in oral mucosa cells and exfoliated bladder cells from smokers and non-smokers (26;37). This may indicate that the low dose sensitivity in human samples may be as low as 1 adduct per 10^8 nucleotides.

Specificity: Dr. Santella has performed a number of experiments to demonstrate the specificity of the assay for PAH-DNA adducts. Oral mucosa cells from a smoker and a non-smoker were stained with a non-specific antiserum recognizing 8-methoxypsoralen-induced DNA damage. The sample from the smoker had a staining intensity equivalent to that in the untreated 10T½ cells, and the staining intensity in the sample from the non-smoker had even lower levels of staining (26). Samples pretreated with DNase or analyzed without the primary antibody showed staining levels at or near the background level (26). Finally, pre-absorption of the primary antiserum with BPDE-I-DNA prior to sample analysis reduced staining levels to background (26). In total these experiments demonstrate that the assay is specific for PAH-DNA adducts.

Reproducibility: The reproducibility of the assay was estimated in two ways. The first was to assay tissue slices from a large breast tumor that had been fixed and embedded into multiple paraffin blocks. The coefficient of variability was 0.25 (n=9). The second estimation was made by analyzing fixed, paraffin-embedded normal lung tissue sections from a rat fed benzo[a]pyrene. The coefficient of variability for these samples was 0.17 (n=4).

The reviewers also stated that the assay "needs to be validated against standard methods for measuring adducts". There is no "gold standard" for measuring PAH-DNA adducts in fixed paraffin-embedded tissues. However, Dr. Santella has treated 10T½ cells in culture with 0, 5, 10, 20 and 40 uM radiolabeled BPDE[2] and has compared immunohistochemical staining intensity with specific activity (26;37). There was a very strong correlation between staining and adduct levels measured by specific intensity ($r=0.99$, $p<0.001$)(37) (26).

Finally, one of the reviewers questioned the use of Optical Density units instead of adduct levels as the measurement. Data have been reported in Optical Density Units as read from the CAS 200

[2] 7R,8S-dihydroxy-9S,10R-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, the adduct forming metabolite of benzo[a]pyrene.

cell analysis system in the absence of a relevant standard curve. One of the barriers to developing a standard curve for this assay is creating tissue specimens with known levels of PAH-DNA adducts. The 10T½ cells discussed above might be useful in this regard but creating the complex mixtures of adducts thought to exist in human tissues is extremely difficult. The use of Optical Density Units rather than antigen concentrations is a standard practice in studies using quantitative immunhistochemistry. Dr. Santella has published five papers on quantitative immunohistochemistry that reported results in Optical Density Units, four of which analyzed either PAH- or 4-aminobiphenyl-DNA adduct levels (25;26;37-39). Further there are numerous methodologic papers on the calculation and use of Optical Density Units in quantitative immunohistochemistry and research papers reporting immunohistochemistry results using this metric (e.g., see (40-43).

d. *p53* Analysis

Immunohistochemical techniques have been used to initially screen for *p53* mutations. While wild type p53 has a very short half-life, many mutant p53 proteins have an increased stability leading to an accumulation of protein that is detectable using immunohistochemical techniques (29;44). Immunohistochemical detection has been found to correlate well with SSCP techniques for mutant detection (45). There is a strong correlation between mutation detection by SSCP and immunohistochemical analysis for p53 protein accumulation and we don't expect the change in techniques to alter the conclusions of the study. As discussed in the year two report, we are working with Affymetric Inc. (Santa Clara, California) to use their gene chip technology to analyze samples for mutational spectra. We are currently analyzing the results from the first batch of 22 cases.

In the literature, samples are scored positive or negative for *p53* accumulation based on the intensity of staining, the percentage of cells that stain positive for p53, or a combination of these two measures (32;45-47). Both of these indices can be measured on the CAS 200 system by using two different software applications: the Cell Measurement Program (CMP) that measures the intensity of staining in optical density units, and the Quantitative Nuclear Antigen Program (QNAP) which measures the percentage of nuclei stained. In year two the samples were scored for intensity of staining only. To make our analysis more comprehensive, in year three we scored samples using both methods. In year three we established procedures for using QNAP in our laboratory and began scoring samples using this software. We intend to re-score year two samples with QNAP; and by the end of the study we will have scored all of the samples by both methods.

Immunohistochemical analysis of p53 is ongoing as tissue blocks are released to us from the Pathology Department. In year two, 62 samples were analyzed for p53 using CMP. In year three an additional 64 samples (20 benign, 18 tumor, and 26 tumor adjacent) were stained and analyzed with QNAP, and scoring with CMP is in progress. Additionally another 50 are being stained. Thus far, of the 126 samples analyzed, 42 (33%) were positive for elevated levels of p53 protein. Considering the mix of tissues analyzed, this is consistent with the published literature (28;29). The remaining samples will be analyzed during year four. In related work, in year three, 33 samples from 20 individuals from LIJMC were analyzed for p53 using both CMP and QNAP. We plan to compare CPMC and LIJMC patients with respect to environmental exposures and p53 levels.

e. Complementary Studies of Genetic Susceptibility and Oncoproteins

As shown in Table 3., using other funds we have analyzed stored samples for genetic susceptibility markers (in white blood cells) and oncoproteins (in tissue). These biomarkers complement our goal of testing whether PAH-, smoking related- and HA-DNA adduct levels are associated with breast cancer status. The genetic susceptibility markers may modulate the relationship between exposure and adduct levels and thus may modify risk relationships (20). The expression of oncoproteins may represent other potential steps along the pathway from environmental exposure to breast cancer. This possibility can be investigated by assaying for these markers in tumor tissue and comparing marker status to potential environmental risk factors in a case-case analysis(48).

i. Polymorphisms in Metabolic Genes

In year two, white blood cell DNAs from our breast cancer cases and controls were analyzed for polymorphisms in genes that mediate the metabolism/detoxification of the environmental carcinogens under study (49-55). In year two, 120 subjects were genotyped for the acetylator genotype (NAT2 slow vs. fast); 134 subjects were genotyped for the glutathione transferase M1 polymorphism (GSTM1 deleted vs. present); and 124 subjects were genotyped for the MspI polymorphism in the CYP1A1 gene (rare allele present vs. wild type). These polymorphisms have previously been associated with increased carcinogen-DNA adduct levels and/or increased breast cancer risk in exposed individuals (55-57). Thus far, the following prevalences of "at risk" genotypes have been found: the slow acetylator genotype, 63%; the rare CYP1A1 allele, 6%; and the deleted GSTM1 locus, 43%. When complete, the genotype dataset will be combined with our case-control and adduct data and will be used to analyze possible gene-environment interactions in breast cancer development. No further genotype assays were performed in year three.

ii. Oncoprotein Overexpression: *cyclin D1* and *erbB-2*

We have analyzed samples for several additional oncoprotein markers. These markers will used to further characterize the cases into potentially more etiological homogeneous sub-groups in which there may be differing associations between risk factors and disease (48;58).

aa. Cyclin D1

The *Cyclin D1* gene is thought to be a proto-oncogene, which, when aberrantly expressed, leads to loss of normal growth control (59;60). *Cyclin D1* overexpression is commonly seen in squamous cell carcinomas of the head, neck, lung and esophagus, as well as in breast carcinomas (59-62). Using immunohistochemical techniques, *cyclin D1* protein overexpression can be detected in paraffin embedded breast tumor specimens with a prevalence of 50-60% (60;60;62;62;63;63).

In year two we analyzed 41 tissue samples (15 benign, 11 normal adjacent, 15 tumor) from 32 of the case-control study subjects for *cyclin D1* protein overexpression using the CAS system and the CMP application. In year three an additional 26 samples were analyzed (10 benign, 8 normal adjacent, 8 tumor) using both the CMP and QNAP applications. Using a cutoff of two standard deviations above the mean optical density in the benign samples, 8 of the 67 samples were found to strongly overexpress *cyclin D1*. This prevalence of overexpression is lower than generally reported in the literature (60;62;63). By the end of next year, all of the samples will have been scored on the CAS system by the CMP and QNAP applications.

In year three, 25 samples (10 Benign; 8 tumor; and 7 normal adjacent)from LIJMC were analyzed for *cyclin D1* expression using the CMP and QNAP applications.

bb. *erbB-2* in Tissue

The *erbB-2* (Her-2 or neu) proto-oncogene codes for a transmembrane receptor-like protein with tyrosine kinase activity that appears to have a pathophysiologic role in the growth and malignant progression of mammary cells (64). The *erbB-2* p185 oncoprotein shows extensive structural similarity to the p170 epidermal growth factor receptor (EGFR) and for this reason is thought to be a growth factor receptor (64). *ErbB-2* is amplified in 25-30% of human primary breast cancers; and amplification is linked to increased *erbB-2* expression in tumors (65-68).

In year two, 42 samples were analyzed for *erbB-2* using a semi-quantitative procedure and 21% of them were found to strongly overexpress *erbB-2*, which is consistent with the literature (65-68). In year three, a fully quantitative procedure was established using the CAS 200 image analysis system, *erbB-2* calibration slides and protein measurement with the Quantitative Oncogene Product application [CAS]. This system determines the amount of protein expressed per cell (picograms *erbB-2*/cell). Thirty-one samples from LIJMC were analyzed for *erbB-2* with this method. The mean protein level in the LIJMC samples was 0.24 pg/cell with a standard deviation of 0.36 pg/cell. We intend to analyze all of the samples using the new quantitative approach.

f. Pilot Laboratory Studies

One of the aims of this study was to create a sample bank that would support future "spin-off" studies. Portions of these samples have been used in pilot studies to support current and future grant proposals for new research using this sample bank. Data from these pilot studies have been analyzed with reference to case-control status so that preliminary results may be presented in future grant applications and to provide data for power calculations.

i. Assay Development: Detection of *Cyclin D1* in Blood Samples

In year three, we began developing an enhanced chemi-luminescent Western blot assay to detect *cyclin D1* in blood samples. The goal of this pilot study is to generate data to support a proposal to analyze paired tissue and blood samples for *cyclin D1*. We are investigating whether analysis of *cyclin D1* in blood samples can be used as a marker of a woman's breast cancer status. This project is analogous to past work showing that other oncoproteins (e.g. *erbB-2* and *p53*) that are often overexpressed in breast tumors can be detected in blood samples from breast cancer patients. It has been shown that increased levels of these proteins in blood samples can predict breast cancer status [see description of *ras p21* work below](69-71). We are currently using samples spiked with *cyclin D1* protein to set up optimal Western blot conditions to achieve sharp bands and a good spread of bands.

ii. *ras* p21 Levels in Blood Samples

In a related pilot project conducted in year two we used an existing technique for detecting *ras* p21 protein in blood samples to assay stored samples. It has been shown in other cancers that *ras* p21 can be detected in blood samples from patients who have tumors that have mutated or overexpressed *ras* (72-74). Since breast tumors commonly overexpress *ras* p21, we investigated whether breast cancer patients exhibited increased blood levels of *ras* and whether these increased levels predicted case-

control status. The development of bloodborne markers that can predict breast cancer status may allow for new strategies to detect breast tumors and could be useful in post-surgical follow-up of patients (69;70;75).

Using the sample bank from the present study, 94 blood plasma samples were analyzed by Western blot and computer aided image analysis of the bands. *Ras* p21 levels, measured as the integrated pixel units (IPU) of a cross-section of the stained bands, were significantly higher in plasma samples from case subjects compared to samples from BBD and healthy control subjects (cases 13.63 IPU n=34; BBD controls 5.65 IPU n=26; and healthy controls 5.24 IPU n=34, p=0.03). Samples were also scored as having elevated versus non-elevated *ras* p21 levels by comparing the staining to normal control samples run on each gel. Twenty nine percent of the case samples and 12% and 13% respectively of the healthy and BBD control samples had elevated levels of *ras* p21. After controlling for age and ethnicity, the presence of elevated *ras* levels in blood plasma significantly predicted case-control status (OR=3.8, p=0.04).

In year three we applied for and received additional funding to establish a quantitative immunohistochemical technique (74) to analyze *ras* p21 levels in tissue samples. The grant also provides funds to analyze expression of *ras* p21 in tissue samples from the case and BBD subjects whose blood samples have been analyzed for *ras* p21. This will allow us to determine how well blood levels are correlated with tissue levels and whether factors such as tumor size and stage influence the relationship.

iii. Blood DDE Levels Among Latina Women in Northern Manhattan

A portion of our present breast cancer study sample is drawn from a large Latina population that immigrated to the United States from the Dominican Republic and settled in Northern Manhattan. Many of these subjects recall intensive government programs of DDT application that included the spraying of homes and aerial spraying of agricultural areas. Since several prior studies have found an association between blood levels of DDE (a metabolite of DDT) and breast cancer, it was of interest to determine whether these intensive exposures have led to increased blood DDE levels in this portion of our study population. In year three, under separate funding, blood plasma samples from 19 healthy controls, 15 benign breast disease controls and 14 cases were analyzed for DDE levels using gas chromatography with electron detection. DDE levels in these Dominican subjects ranged from 0.2 - 113.2 ppm with a mean of 18.3 ppm. No significant differences could be detected between cases and controls, possibly due to the small number of subjects.

DDE levels in these subjects were compared to levels previously measured in 24 healthy non-Dominican women from the New York Metropolitan region who were treated at the Columbia-Presbyterian Medical Center for breast cysts. DDE levels were 3.22 times higher in the Dominican women than in the non-Dominican women (14.76 ppm vs. 4.56 ppm, p=0.005). Additionally, DDE levels were significantly associated with the number of years the women had lived in the Dominican Republic, with age and with the number of months the women breast feed their children. The increased body burden of DDE seen in Dominican women suggests that they would be a good model population in which to study the possible link between DDE and breast cancer.

The blood samples were also analyzed for DDT, PCB (total and 21 congeners), hexochloro-benzene, and Mirex (1,200 determinations in total). Statistical analyses of these compounds has not yet been completed.

C. Conclusion

Patient recruitment continued to be highly successful in year three, with a total of 384 subjects enrolled thus far, with approximately equal numbers of cases and controls. Patient recruitment will continue through January 1998 and is aimed at completing the frequency matching and balancing out the age distribution in the cases. Questionnaire data are being entered into the database as the patients are enrolled and data entry and cleaning are expected to be completed by March of 1998. We do not anticipate difficulty in completing analyses on blood and tissue samples. We plan to complete the laboratory portion of the study by June of 1998. The remainder of year four will be spent on data analysis and manuscript preparation.

We anticipate that this study will provide valuable information on the role of PAH and cigarette smoke constituents in the etiology of breast cancer. The identification of environmental risk factors could provide novel opportunities to prevent breast cancer.

Table 2. Progress on Assays for Major Hypotheses

ASSAY	COMPLETED IN	COMPLETED IN YEAR 3	TOTAL TO DATE
PAH-DNA in MNWBC	75	75	150
HA-DNA in MNWBC	75	53	128
Smoking Related Adducts in MNWBC	75	75	150
PAH-DNA in Fresh Tissue (^{32}P)	0	18	18
PAH-DNA in Paraffin Tissue (IH)	15	27	42
P53 in Tissue (IH)	62	64	126

Abbreviations: MNWBC, mono-nuclear white blood cells; ^{32}P, ^{32}P-postlabeling; IH, immunohistochemistry.

Table 3. Assays Performed for Complementary and Pilot Studies

ASSAY	COMPLETED IN YEAR 2	COMPLETED IN YEAR 3	TOTAL TO DATE
NAT2	120	0	120
GSTm1	134	0	134
Cyp1A1 MSP1	124	0	124
Cyclin D1	41	51	92
ErbB-2	42	31	73
Ras p21	94 (blood)	0	94
Organochlorines[1]	0	1,200	1,200

1 Includes analyses for DDE, DDT, 21 different PCB congeners, hexochloro-benzene and Mirex on 48 subjects.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900

Encl